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Purkinje Fibers of the Avian Heart Express a Myogenic Transcription Factor Program Distinct from Cardiac and Skeletal Muscle

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A rhythmic heart beat is coordinated by conduction of pacemaking impulses through the cardiac conduction system. Cells of the conduction system, including Purkinje fibers, terminally differentiate from a subset of cardiac muscle cells that respond to signals from endocardial and coronary arterial cells. A vessel-associated paracrine factor, endothelin, can induce embryonic heart muscle cells to differentiate into Purkinje fibers both *in vivo* and *in vitro*. During this phenotypic conversion, the conduction cells down-regulate genes characteristic of cardiac muscle and up-regulate subsets of genes typical of both skeletal muscle and neuronal cells. In the present study, we examined the expression of myogenic transcription factors associated with the switch of the gene expression program during terminal differentiation of heart muscle cells into Purkinje fibers. *In situ* hybridization analyses and immunohistochemistry of embryonic and adult hearts revealed that Purkinje fibers up-regulate skeletal and atrial muscle myosin heavy chains, connexin-42, and neurofilament protein. Concurrently, a cardiac muscle-specific myofibrillar protein, myosin-binding protein-C (cMyBP-C), is down-regulated. During this change in transcription, however, Purkinje fibers continue to express cardiac muscle transcription factors, such as *Nkx2.5*, *GATA4*, and *MEF2C*. Importantly, significantly higher levels of *Nkx2.5* and *GATA4* mRNAs were detected in Purkinje fibers as compared to ordinary heart muscle cells. No detectable difference was observed in *MEF2C* expression. In culture, endothelin-induced Purkinje fibers from embryonic cardiac muscle cells dramatically down-regulated *cMyBP-C* transcription, whereas expression of *Nkx2.5* and *GATA4* persisted. In addition, *myoD*, a skeletal muscle transcription factor, was up-regulated in endothelin-induced Purkinje cells, while *Myf5* and *MRF4* transcripts were undetectable in these cells. These results show that during and after conversion from heart muscle cells, Purkinje fibers express a unique myogenic transcription factor program. The mechanism underlying down-regulation of cardiac muscle genes and up-regulation of skeletal muscle genes during conduction cell differentiation may be independent from the transcriptional control seen in ordinary cardiac and skeletal muscle cells.

Key Words: **cardiac conduction system; Purkinje fiber; muscle transcription factors; myofibrillar protein; chicken embryo; heart development.**

INTRODUCTION

Contraction of the heart in higher vertebrates is regulated by conduction of electrical excitation through the cardiac conduction system (Tawara, 1906; Goldenberg and Rothberger, 1936; Bozler, 1942). A pacemaking action potential is generated at the sinoatrial node and then guided across the atrial chambers to the atrioventricular node. After a brief delay, pacemaking impulses are rapidly propagated along the atrioventricular bundle and Purkinje fiber network and, finally, spread into ventricular muscle. The Purkinje fiber network, the most distal component of the conduction system, is specialized to transmit electrical impulses to the apex of the heart, initiating synchronous apical-to-basal ejection of blood from both ventricular chambers (Purkinje, 1845; Kolliker, 1902; Tawara, 1906).

In the avian heart, Purkinje fibers are characterized as a cellular network ramifying along the subendocardium and periarterial bed (Davies, 1930; Vassal-Adams, 1982; Lamers *et al.*, 1991; Gourdie *et al.*, 1995). Using retroviral cell tagging procedures (Mikawa *et al.*, 1992a,b, 1996), we have

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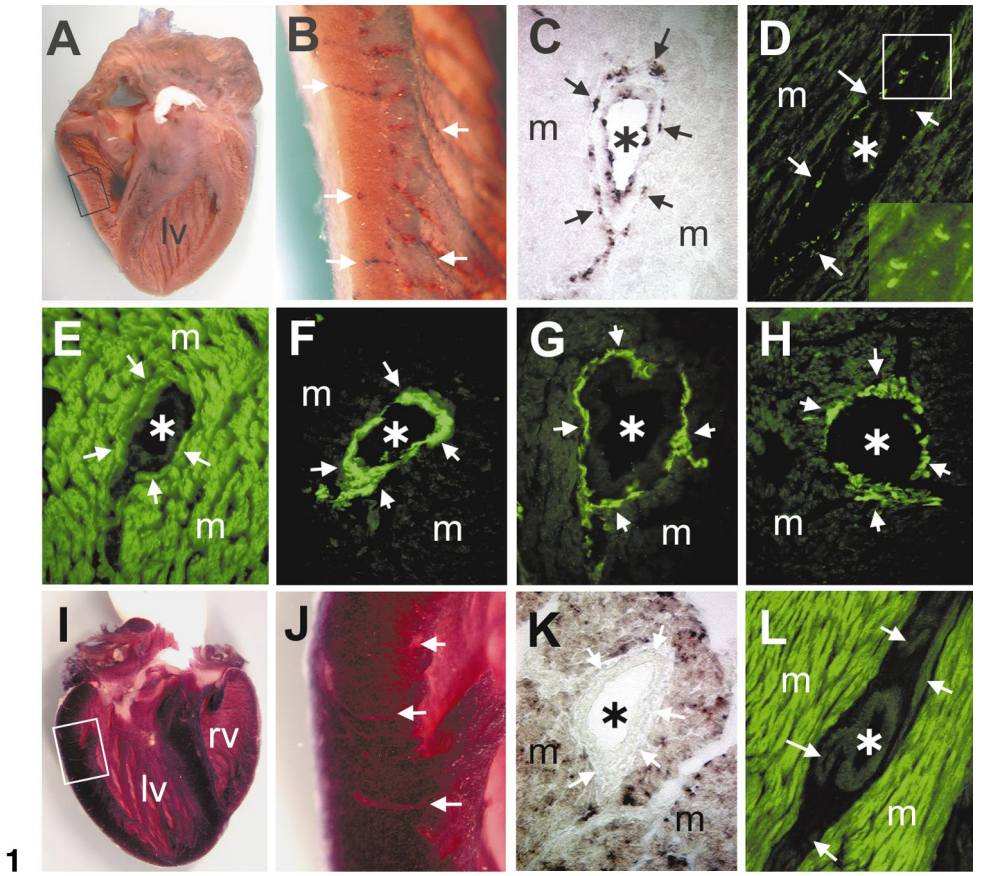
TABLE 1
Probes and Conditions for PCR Analysis

Gene	Primers	Product size (bp)	Annealing temp. (°C)	Cycles
MyBP-C	5′-ACCGAGTGGTTCTTTTGGGG-3′ 5′-TTCTACTGGAGCAGGCAAC-3′	477	56.5	30
Cx42	5′-GTCCGCCCCACAGGTAGAAA-3′ 5′-GTCCCACGGGCTGAGAACTT-3′	580	50	30
Myf5	5′-GAGGGAATCGCTGCATTGTG-3′ 5′-CCTGCTCTTACCATCACATCGG-3′	578	55	30
MRF4	5′-GAAGAGGCGGTGGAGAAATGAG-3′ 5′-AAAAGGATGGGGGTGGTCTG-3′	164	55	30
MEF2C	5′-GATTTCCACTCTCCTGT-3′ 5′-TTACCATGGGACATCT-3′	314	55	40
MyoD	5′-CGTGAGCAGGAGGATGCATA-3′ 5′-GGGACATGTGGAGTTGTCTG-3′	280	55	30
Myogenin	5′-AGCCTCAACCAGCAGGAG-3′ 5′-TGCGCCAGCTCAGTTTTGGA-3′	284	55	30
Nkx2.5	5′-CCTTCCCCGGCCCCTACTAC-3′ 5′-CTGCTGCTTGAACCTTCTCT-3′	225	50	30
GATA4	5′-TTCAGAAGGGCGAGAATGTGTC-3′ 5′-CCTGCTGGCGTCTTTGTTTTG-3′	366	56	30
GAPDH	5′-ACGCCATCACTATCTTCCAG-3′ 5′-CAGCCTTCACTACCCTCTTG-3′	578	55	30

previously shown that both subendocardial and periarterial Purkinje fibers differentiate from contractile cardiomyocytes (Gourdie *et al.,* 1995; Cheng *et al.,* 1999). The results provided evidence that the mechanism by which Purkinje fibers differentiate is direct conversion of heart muscle cells into conducting cells (Mikawa and Fischman, 1996; Gourdie *et al.,* 1999). Recruitment of Purkinje fibers and their branching pattern in the ventricular wall appear to be instructed by local cues derived from cells of the endocardium and developing arteries (Hyer *et al.,* 1999; Takebayashi-Suzuki *et al.,* 2000). A survey of vessel-associated paracrine factors showed that endothelin (ET) (Yanagisawa *et al.,* 1988; Masaki *et al.,* 1991; Yanagisawa, 1994) is capable of inducing embryonic myocytes to differentiate into Purkinje fibers both *in vivo* (Takebayashi-Suzuki *et al.,* 2000) and *in vitro* (Gourdie *et al.,* 1998).

Purkinje fibers are distinguished from heart muscle cells by a distinct pattern of gene expression (reviewed in Mikawa and Fischman, 1996; Schiaffino, 1997; Moorman *et al.,* 1998; Welikson and Mikawa, 2000). They up-regulate Cx42, a conduction cell-specific gap-junction protein (Gourdie *et al.,* 1993; Bastide *et al.,* 1993; Delorme *et al.,* 1995), unique ion channels (Callewaert *et al.,* 1986; Hagiwara *et al.,* 1998), and genes typically expressed in neuronal cells (Gorza *et al.,* 1988, 1989, 1994; Gourdie *et al.,* 1995; Takebayashi-Suzuki *et al.,* 2000). In addition, conduction cells induce a unique set of myofibrillar protein genes, such as slow-twitch skeletal muscle myosin heavy chain (sMyHC) (Sartore *et al.,* 1978; Gonzalez-Sanchez and Bader, 1985), atrial myosin heavy chain (aMyHC) (de Groot *et al.,* 1987), and skeletal muscle-type myosin binding protein-H (MyBP-H) (Alyonycheva *et al.,* 1997). Purkinje fibers also down-regulate heart muscle-specific myofibrillar proteins, such as troponin (Gorza *et al.,* 1994) and myosin binding protein-C (cMyBP-C) (Gourdie *et al.,* 1998; Takebayashi-Suzuki *et al.,* 2000), which are essential for normal heart muscle contractility (Watkins *et al.,* 1995; Bonne *et al.,* 1995). Promoter analysis of the *desmin* gene revealed that a skeletal muscle enhancer element functions in the cardiac conduction system but not in ordinary heart muscle cells (Li *et al.,* 1993). Together with the cell lineage data, these studies suggest that Purkinje fiber differentiation involves a switch of myofibrillar protein gene expression.

Transcription factors responsible for conduction cell differentiation are largely unknown. *Msx-2*, a homeobox domain gene homologous to the *Drosophila* muscle segment homeobox gene, has been found to be expressed transiently in conduction cell progenitors in the developing chick hearts (Chan-Thomas *et al.,* 1993). In addition, *Nkx2.5* (Komuro and Izumo, 1993), the mammalian homologue of the *tinman* gene in *Drosophila* (Bodmer, 1993), has been linked to the conduction system (Schott *et al.,* 1998): Mutations in human *NKX2.5* result in an autosomal dominant atrial septal defect, an inherited disease associated with atrioventricular conduction delays. In the mouse, *Nkx2.5* is expressed in myocardial cells both in embryonic and adult hearts (Komuro and Izumo, 1993). *Nkx2.5* knockout in mice results in embryonic lethality at the tubular heart stage (Lyons *et al.,* 1995). Nkx2.5 can bind to GATA4, a zinc finger domain protein (Molkentin *et al.,* 1997). These



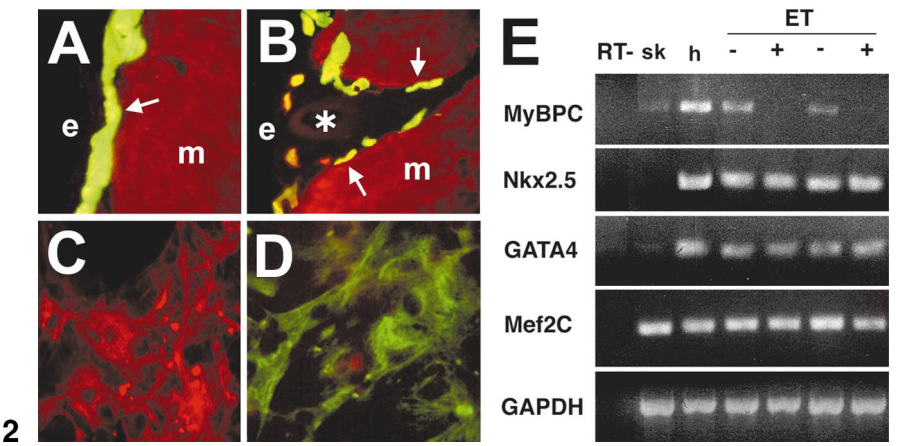


FIG. 1. Whole-mount (A, B, I, and J) and section (C and K) *in situ* hybridization (brownish purple signals) and immunostaining (D–H and L; green signals) of embryonic (A, B, E–J) and adult (C, D, K, and L) chicken hearts with probes for Cx42 (A–D), sarcomeric MyHCs (E), slow skeletal muscle MyHC (F), atrial MyHC (G), neurofilament (H), and cMyBP-C (I–L). (B, J) Higher power views of boxed areas in (A) and (I), respectively. Inset in (D) is a higher magnification of the boxed area. Rv, right ventricle; lv, left ventricle; m, myocytes; arrows, Purkinje fibers; asterisks, lumen of arteries. cMyBP-C is expressed predominantly in ordinary heart muscle cells, and absent in Purkinje fibers.

FIG. 2. ET-dependent induction of Purkinje fiber marker genes *in vitro*. Double immunostaining of sections from mature hearts (A, B) and monolayer culture of E3 myocytes unexposed (C) and exposed (D) to ET (10^{-7} M) for 5 days, probed for cMyBP-C (red signal) and sMyHC (green signal). Arrows, subendocardial (A) and periarterial (B) Purkinje fibers; asterisks, lumen of arteries; m, myocytes; e, endocardium. (E) RT-PCR analysis of transcription factors in the skeletal muscle (sk), whole heart (h), and embryonic myocytes cultured with (+) or without (−) ET. RT−, Control reaction without RT.

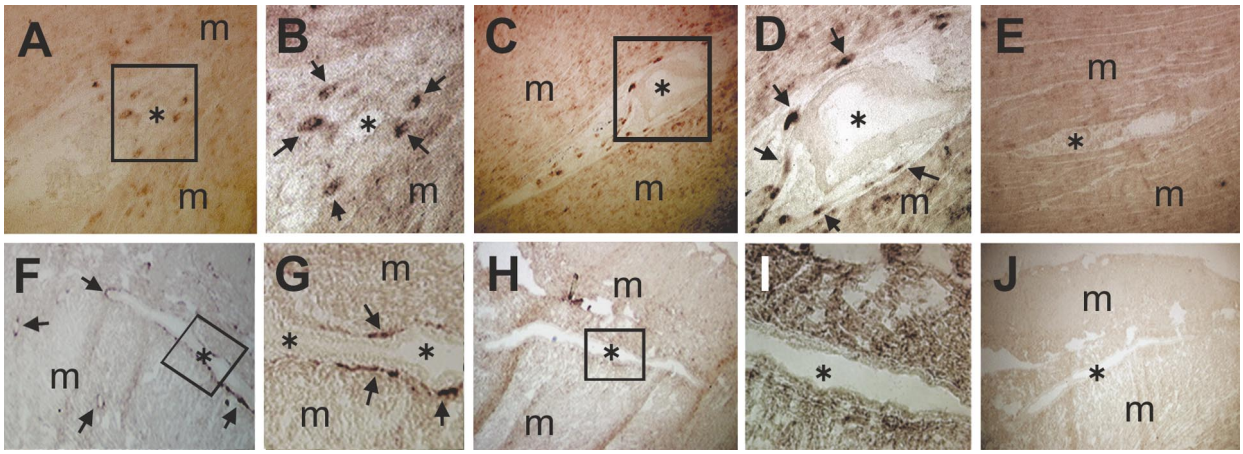


FIG. 3. Section *in situ* hybridization of adult (A–E) and embryonic (F–J) ventricles with probes for Nkx2.5 (A, B, F, G), GATA4 (C, D, H, I), and Mef2C (E, J). (B, D, G, I) High-power views of the boxed areas in A, C, F, and H, respectively. Arrows, Purkinje fibers; asterisks, lumen of arteries and arterioles.

two transcription factors activate cooperatively the expression of atrial natriuretic factor (ANF) (Dorocher *et al.*, 1997; Lee *et al.*, 1998) that is rich in both the atrium and the ventricular conduction system (Wharton *et al.*, 1988; Hansson and Forsgren, 1993). These data, however, do not explain the mechanism of down-regulation of ventricular muscle-specific genes in Purkinje fibers or how genes typical of neuronal or skeletal muscle lineages are up-regulated in conduction cells.

Expression of many of the skeletal muscle-specific proteins are regulated by the bHLH family of myogenic transcription factors (reviewed in Olson and Srivastava, 1996), such as myoD, Myf-5, myogenin, and MRF-4. These factors, which are solely expressed in skeletal muscle cells and their precursors, bind to the cis-element, E-box, in the upstream region of muscle structural genes (Fujisawa-Sehara *et al.*, 1992). The activation of muscle genes by bHLH transcription factors is believed to require binding to cofactors, such as the myogenic enhancer factor 2 (Mef2) family (Kaushal *et al.*, 1994). While cardiac muscle cells do not express any of the members of the *myoD* gene family, they express Mef2s (Edmondson *et al.*, 1994; Goswami *et al.*, 1994). In the heart, GATA factors have been shown to interact with Mef2s (Morin *et al.*, 2000).

In the present study, we examined expression of these myogenic transcription factors in Purkinje fibers of the embryonic and adult chicken heart as well as in embryonic myocytes converted experimentally into conduction cells with ET. Immunohistochemical, *in situ* hybridization, and RT-PCR analyses of Nkx2.5, GATA4, myoD, myogenin, MRF4, Myf5, and Mef2C are presented. The data show that both *bona fide* and ET-induced Purkinje fibers continue to express or up-regulate the cardiac-specific transcription factors, Nkx2.5 and GATA4, while at the same time cMyBP-C is down-regulated. In addition, although Purkinje fibers express only a trace amount of myoD and no detectable transcripts of MRF4 and Myf5, they still induce skeletal muscle myofibrillar proteins. These results suggest that down-regulation of cardiac muscle genes and up-regulation of skeletal muscle genes during conduction cell differentiation, may be regulated by a unique myogenic transcriptional program distinct from those seen in ordinary cardiac and skeletal muscle cells.

MATERIALS AND METHODS

Histochemistry and Immunohistochemistry

Embryos and hearts were removed and fixed by immersion overnight in 2% paraformaldehyde/PBS at 4°C. Frozen sections of ethanol fixed hearts were prepared as described (Gourdie *et al.*, 1995). Primary antibodies used in the present study were ALD58 against sMyHC (Gonzalez-Sanchez and Bader, 1985), MF20 against sarcomeric MyHC (Bader *et al.*, 1982), anti-β-Gal (5 prime 3 prime Co), EAP3 against neurofilament EAP300 (Gourdie *et al.*, 1995), the Guinea pig-318 anti-Connexin 40/42 (Gourdie *et al.*, 1998), C315 against cMyBP-C (Yasuda *et al.*, 1995), anti-aMyHC antibody (Sanders *et al.*, 1984), M-318 against myoD (Santa Cruz Biotech), and M-225 against myogenin (Santa Cruz Biotech). Immunofluorescent labeling was undertaken according to methods detailed previously (Gourdie *et al.*, 1995, 1998; Hyer *et al.*, 1999; Takebayashi-Suzuki *et al.*, 2000).

Cell Culture

Myocytes were isolated from the ventricular segment of E3 chick embryos by a conventional method, and maintained as matrix-adherent cultures (Mikawa *et al.*, 1992; Gourdie *et al.*, 1998) on four chambered-slide plates at a density of $\sim 2 \times 10^4$ cells per well (Takebayashi-Suzuki *et al.*, 2000). Myocyte cultures were then exposed to 10^{-7} M of ET-1 (Sigma). Culture medium containing ET-1 was replaced every 12 h thereafter. Control cultures were

treated in the same manner except that 1% BSA was used instead of ET-1. Three to five days later, one group of cultures was fixed with 70% ethanol for 10 min at −20°C and processed for immunohistochemistry, while the other group was harvested for RT-PCR analysis.

RT-PCR Analysis

Total RNA was extracted from cultured cells, E16 pectoral muscle, and E16 heart with Trizol reagent (Gibco BRL). cDNA was synthesized from 1 μg of total RNA with AMV reverse transcriptase (Roche) using random primers. PCR was carried out as described (Schultheiss *et al.,* 1995) with a slight modification: dNTPs and 5% deionized formamide were added throughout PCR. Thermal cycling was performed for 30 cycles, except for amplification of *Mef2C* which required 40 cycles. Primers of *Myf5*, *MRF4*, and *GATA4* are listed in Table 1, while those of *cMyBP-C* (Yasuda *et al.,* 1995) and *Cx42* (Beyer, 1990) were described previously (Takebayashi-Suzuki *et al.,* 2000). Primers of *Mef2C* were designed according to Goswami *et al.* (1994), and those of *Nkx-2.5*, *MyoD*, *myogenin*, and *GAPDH* were synthesized as described (Schultheiss *et al.,* 1995). A total of 10 μl of PCR products were loaded on an agarose gel and stained with ethidium bromide. PCR products of the expected size were TA-subcloned into pCRII (Invitrogen) or pGemT Easy Vector (Promega), and sequenced in both directions, as described (Takebayashi-Suzuki *et al.,* 2000). The resulting plasmids of *Cx42*, *cMyBP-C*, *myogenin*, *myoD*, and *Mef2C* were used as templates for RNA probe.

RNA Probes

DIG- and radiolabeled RNA probes were generated from linearized plasmids by using standard riboprobe protocols. In short, *myogenin* probe was transcribed with T7 RNA polymerase from *Xba*I-digested plasmid DNA, while *Mef2C* probe was synthesized from *Bam*HI-linearized plasmid DNA. Probes of *MyBP-C* and *Cx42* were prepared as described (Takebayashi-Suzuki, 2000). Probes of *GATA-4* and *Nkx2.5* were transcribed according to Laverriere *et al.* (1994), while those of *myoD* were synthesized as described (Bober *et al.,* 1994). *VMHC1* riboprobes were generated directly from pVMHC1 (Bisaha *et al.,* 1991) according to Yutzey *et al.* (1994). After transcription, template DNA was digested with DNase and riboprobes were hydrolyzed into ~300-bp fragments.

In Situ Hybridization

Fertilized chicken eggs were incubated at 38°C and staged as described (Hamburger and Hamilton, 1951). Whole embryos or hearts were fixed with 4% paraformaldehyde/PBS and processed for whole-mount (Wilkinson, 1992) or section *in situ* hybridization, according to methods previously described (Takebayashi-Suzuki *et al.,* 2000). In short, fixed embryos and hearts were treated with proteinase K for 20 min at the concentrations of 10 μg/ml for E1–E4 embryos and E7–E10 hearts, and 70 μg/ml for E15–E18 hearts. After postfixation with 4% formaldehyde and 0.1% glutaraldehyde in PBS, samples were preincubated with the standard hybridization mixture (Henrique *et al.,* 1995) for 1 h at 65°C, and reacted overnight at 65°C with 0.1–0.5 μg/ml DIG-labeled RNA probes. Some stained embryonic hearts were further processed for histochemistry, as described (Takebayashi-Suzuki *et al.,* 2000).

Frozen sections were treated with RNase A, omitting proteinase K treatment. After 65°C washes and blocking, the samples were incubated overnight at 4°C with alkaline phosphatase-conjugated anti-DIG antibody (Boehringer), rinsed, and stained with the NBT/BCIP mixture at room temperature until color developed. Paraffin sections (4 μm) were mounted on Superfrost Plus slides (VWR) and processed essentially as described (Duncan, 1994) with the following modifications. Pretreated slides were incubated with prehybridization buffer, containing 50% formamide, 0.3 M NaCl, 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, salmon sperm DNA (500 μg/ml), and yeast tRNA (500 μg/ml), at 65°C for 2 h. After prehybridization, slides were incubated overnight with 5×10^6 cpm of probe in hybridization buffer containing 50% formamide, 0.3 M NaCl, 20 mM Tris–HCl (pH 8.0), 5 mM EDTA, 10 mM Sodium phosphate buffer (pH 8.0), 10% dextran sulfate, 1× Denhardt's and yeast tRNA (500 μg/ml) at 65°C. Slides were washed as described (Duncan, 1994), dehydrated, and exposed to emulsion for 4 weeks.

RESULTS

Change in Myofibrillar Protein Composition during Purkinje Fiber Differentiation

Purkinje fibers were identified and distinguished from ordinary cardiomyocytes by preferential expression of connexin 42 (Cx42), a gap-junctional protein responsible for rapid electrical conduction in both embryonic and adult chicken heart (Figs. 1A–1D). Composition of myofibrillar and cytoskeletal proteins was then examined in Purkinje fibers during their differentiation from contractile myocytes. Myofibrillar and cytoskeletal components examined include cardiac muscle-specific myosin-binding protein (cMyBP-C), slow twitch skeletal muscle myosin heavy chain (sMyHC), atrial myosin heavy chain (aMyHC), and neurofilament (EAP300).

Immunohistochemical inspection of the embryonic myocardium showed that among cells positive for sarcomeric MyHCs (Fig. 1E), developing Purkinje fibers, but not contractile myocytes, uniquely expressed sMyHC and aMyHC (Figs. 1F and 1G). The conduction cells were also positive for neurofilament (Fig. 1H). Undetectable levels of expression of these markers were seen in beating myocytes. Whole-mount *in situ* hybridization analysis revealed that ventricular myocytes ubiquitously expressed cMyBP-C, whereas developing, intramural Purkinje fibers already down-regulated cMyBP-C (Figs. 1I and 1J). Down-regulation of cMyBP-C became more evident in mature conduction cells (Figs. 1K and 1L), as we reported previously (Gourdie *et al.,* 1998; Takebayashi-Suzuki *et al.,* 2000). The data demonstrate that Purkinje fiber differentiation from contractile myocytes involves down-regulation of a cardiac muscle protein in addition to induction of conduction-specific elements and skeletal muscle myofibrillar proteins.

ET-Induced Purkinje Fibers Down-Regulate cMyBP-C but Not Nkx2.5 and GATA4

Down-regulation of cMyBP-C protein was detected in subendocardial Purkinje fibers (Fig. 2A), in addition to periarterial Purkinje fibers (Fig. 2B) as reported previously

(Gourdie *et al.*, 1998). The data now show that cMyBP-C is down-regulated commonly in both subendocardial and periarterial components of Purkinje fiber network. To address whether change in expression of cardiac muscle transcription factors is associated with down-regulation of cMyBP-C, mRNAs of *Nkx2.5*, *GATA4*, and *Mef2C* were examined in ET-induced Purkinje cells. We have previously shown that the down-regulation of cMyBP-C associated with Purkinje fiber differentiation can be induced by exposing embryonic myocytes to ET-1 (Gourdie *et al.*, 1998; Takebayashi-Suzuki *et al.*, 2000). Under our culture conditions, control myocytes maintained the expression of cMyBP-C protein and rarely induced sMyHC, a Purkinje fiber marker (Fig. 2C). In contrast, cells treated with ET-1 dramatically down-regulated cMyBP-C and induced sMyHC (Fig. 2D). Consistent with the immunohistochemical data, ET treatment resulted in significant decrease of the levels of *cMyBP-C* mRNA (Fig. 2E) as seen in differentiation of *bona fide* Purkinje fibers (Figs. 1I–1L). Despite this significant decline of *cMyBP-C* transcripts, no detectable change was observed in the levels of *Nkx2.5*, *GATA4*, and *Mef2C* mRNAs in ET-treated cells (Fig. 2E). These data suggest that ET-induced down-regulation of *cMyBP-C* can occur without transcriptional suppression of *Nkx2.5*, *GATA4*, and *Mef2C*.

Bona Fide Purkinje Fibers Express Nkx2.5 and GATA4

Although ET-induced phenotypes seen above and in our previous reports (Gurdie *et al.*, 1998; Takebayashi-Suzuki *et al.*, 2000) highly resemble those of *bona fide* Purkinje fibers, the underlying transcriptional control between the two systems might be different. To clarify this possibility, expression of cardiac transcriptional factors in *bona fide* Purkinje fibers was examined in embryonic and adult hearts with *in situ* hybridization analysis (Fig. 3). In adult hearts, hybridization signals of *Nkx2.5* (Figs. 3A and 3B) and *GATA4* (Figs. 3C and 3D) transcripts were found in Purkinje fibers at higher levels than those seen throughout ventricular myocytes. While *Mef2C* signals were detected throughout the myocardium, virtually no difference was seen between myocytes and Purkinje fibers (Fig. 3E).

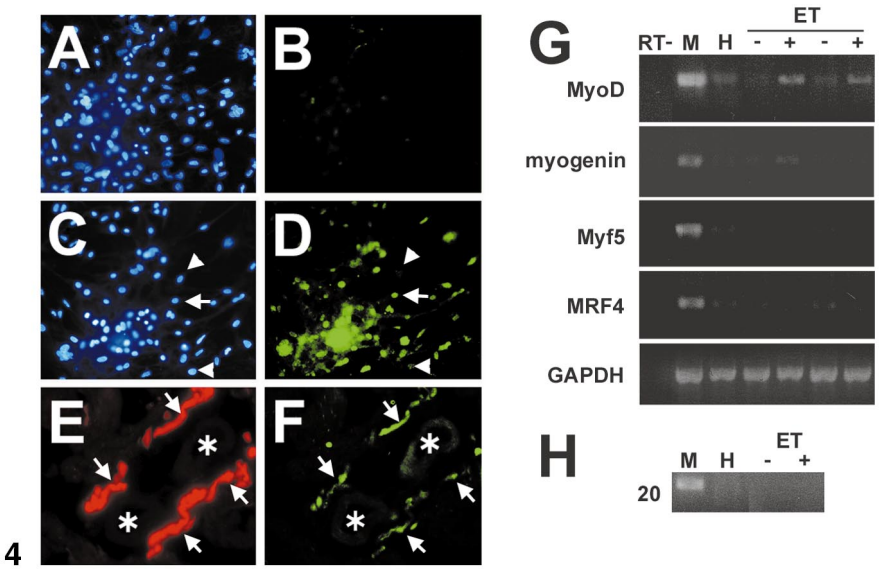
In embryonic hearts, higher levels of *Nkx2.5* signal were detected in developing Purkinje fibers than in contractile myocytes (Figs. 3F and 3G) as seen in the adult heart (Figs. 3A and 3B). In contrast, *GATA4*-signals exhibited similar levels in both contractile myocytes and Purkinje fibers (Figs. 3H and 3G). *Mef2C*-signals were also detected in both cell types with similar intensity (Fig. 3J). Hybridization signals in other tissues such as the coronary vasculature (Fig. 3) were found at the same level as those with sense probes (not shown). These results demonstrate that *Nkx2.5*, *GATA4*, and *Mef2C* continue to be expressed or up-regulated in Purkinje fibers in which *cMyBP-C* is down-regulated. Thus, transcriptional inhibition of these cardiac muscle-transcription factors appears not to be associated with down-regulation of *cMyBP-C* during and after Purkinje fiber differentiation from contractile myocytes.

ET-Dependent Expression of Skeletal Muscle Transcription Factors

Concurrent with down-regulation of cMyBP-C, skeletal muscle-type myofibrillar proteins are induced in Purkinje fibers, as seen in Figs. 1 and 2 and in our previous reports (Alyonycheva *et al.*, 1997; Takebayashi-Suzuki *et al.*, 2000). Although skeletal muscle-type transcription factors have not been identified in heart muscle cells, they may play a role in unique gene expression of Purkinje fibers. To test this possibility, expression of *myoD*, *myogenin*, *Myf5*, *MRF4*, and *Mef2C* was examined in ET-induced and *bona fide* Purkinje fibers.

In the first set of experiments, ET-treated cells in culture were immunostained with antibodies raised for basic helix—loop–helix domains of myoD and myogenin (Figs. 4A–4D). Positive immuno-signals were found in the nuclei of the majority of cells, if not all, that were treated with ET. Immunohistochemistry of the adult ventricular myocardium with the same antibodies also detected a subpopulation of Purkinje fibers (Figs. 4E and 4F). However, in some cases, the immuno-signals were found in the cytoplasm of Purkinje fibers in addition to their nuclei. The results led us to suspect the identity of epitopes that were recognized with the antibodies in the heart. Unfortunately, our attempt at immuno-blot analysis of myoD and myogenin was unsuccessful, perhaps because Purkinje fibers are an extremely minor population within the heart.

As an alternative approach, mRNAs of *myoD* and *myogenin*, as well as *Myf5*, *MRF4*, and *Mef2C*, were examined with RT-PCR analysis (Fig. 4G), using primers listed in Table 1. Expected levels of signals were obtained for all four factors from skeletal muscle mRNA pools. The same size of bands with much weaker signals were amplified from mRNAs of heart muscle and cultured embryonic myocytes (Fig. 4G). Nucleotide sequence analysis confirmed that RT-PCR amplified products from skeletal muscle, cardiac muscle, and cultured embryonic myocytes were identical (not shown). Interestingly, ET-treated embryonic myocytes exhibited a slight increase in *myoD* mRNA (Fig. 4G). In addition, in some cases, a weaker up-regulation of *myogenin* mRNA was detected in ET-treated myocytes (Fig. 4G). No such induction was evident in expression of *Myf5* and *MRF4* mRNAs. These results show that, although embryonic myocytes can regulate the expression of *myoD* in an ET-dependent manner, ET-induced up-regulation of skeletal muscle-type proteins occurs in the absence of detectable *Myf5* and *MRF4* transcription. Furthermore, *myoD* mRNA in the heart and ET-treated embryonic myocytes became detectable with 30 cycles of RT-PCR amplification, but not with 20 cycles which gave rise to robust *myoD* signals in skeletal muscle (Fig. 4H). The data suggest that the expression levels of skeletal muscle transcription fac-



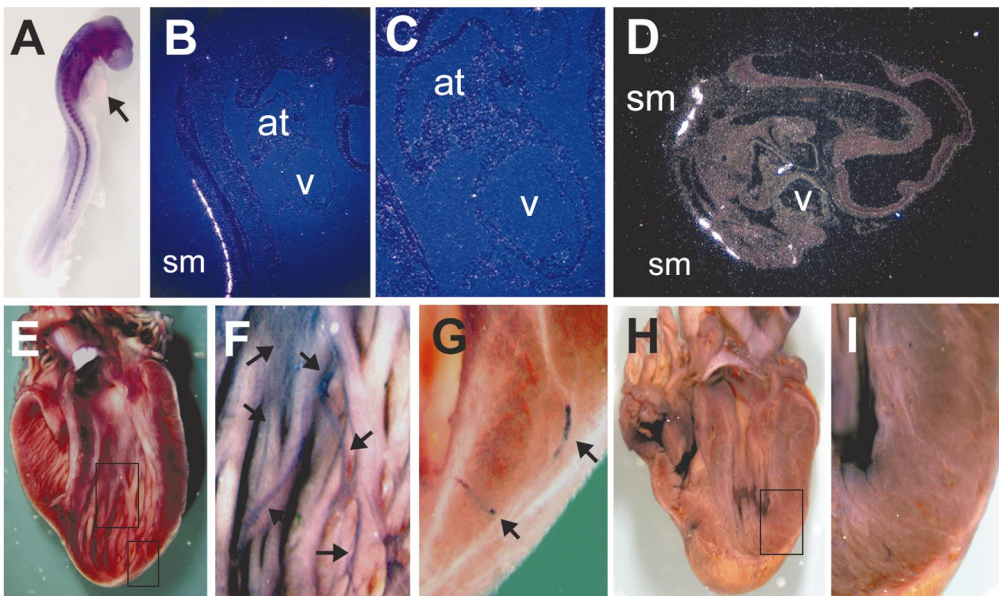


FIG. 4. Control (A, B) and ET-treated (C, D) embryonic myocytes and cryosections of the adult chicken heart (E, F) were doubly stained with DAPI for nuclei (A, C; blue signals) or ALD58 for sMyHC (E; red signals) and antibodies for myoD (B, D, and F; green signals). Arrows and arrowheads indicate myoD-positive and -negative nuclei, respectively. Among antibodies used, only those raised for the bHLH domain of MyoD and myogenin identified positive immuno-signals in ET-induced Purkinje fibers. (G) RT-PCR analysis of transcription factors in the skeletal muscle (sk), whole heart (h), and embryonic myocytes cultured with (+) or without (−) ET, as described in Fig. 2. RT−, Control reaction without RT. (H) Same as (G), except for 20 cycles of PCR.

FIG. 5. Expression pattern of *myoD* in the chick embryonic heart. Riboprobes were generated from pCRII encoding the RT-PCR amplified cDNA of *myoD*. (A) Whole-mount *in situ* hybridization of E3 chick embryo, demonstrating the myoD probe identifies myoD in myotomes, a typical *myoD* expression pattern, but no detectable hybridization signal in the heart (arrow). (B) Section *in situ* hybridization of E3 chick embryo with ^{33}P-labeled antisense riboprobe of *myoD*, confirming significant expression of myoD in somites (sm) but not in the heart (h). (C) High-power view of the heart region of (B) (arrow). At, atrium; v, ventricle. (D) Section *in situ* hybridization with ^{33}P-labeled antisense riboprobe of *myogenin*. (E–G) Whole-mount *in situ* hybridization of E16 chick embryonic heart with myoD riboprobes. (F, G) Higher power views of the boxed areas in (E). Arrows, periarterial Purkinje fibers; asterisks, lumen of arteries. The data show that myoD expression becomes detectable just before skeletal muscle MyHC expression begins. (H) Whole-mount *in situ* hybridization with *myogenin* riboprobes. (I) A high-power view of the boxed area in (H).

tors in ET-induced Purkinje fibers appeared to be significantly lower than those seen in skeletal muscle.

MyoD mRNA in a Subpopulation of Bona Fide Purkinje Fibers

The above results of RT-PCR analysis suggest that *myoD* which is slightly up-regulated with ET in culture may also be expressed in *bona fide* Purkinje fibers of the embryonic heart. To test this possibility, expression of *myoD* was examined in embryonic hearts with *in situ* hybridization analysis (Fig. 5). Robust hybridization signals of *myoD* were found in somites of E3 embryos in whole mount (Fig. 5A). At this stage prior to initiation of Purkinje fiber differentiation, no signals beyond the level with sense probe (not shown) were seen in the heart (Fig. 5A). Essentially the same results were obtained by *in situ* hybridization analysis on histological sections with ^{33}P-labeled *myoD* (Figs. 5B and 5C) and *myogenin* (Fig. 5D) riboprobes: robust hybridization signals were detected in somites and no signals were found in the heart.

In striking contrast, in E16 hearts in which Purkinje fiber differentiation had already begun, hybridization signals of *myoD* were found in a subpopulation of a tissue network ramifying subendocardially (Fig. 5F) and periarterially (Fig. 5G). The relatively weak hybridization signal in *bona fide* Purkinje fibers was consistent with lower levels of *myoD* expression seen in ET-induced conduction cells in culture (Fig. 4G). Under the same hybridization condition, no detectable *myogenin* signals beyond the background level were detected either in Purkinje network or in ordinary myocytes of E16 hearts (Figs. 5H and 5I), although a smaller amount of *myogenin* transcripts was detected by RT-PCR in ET-induced Purkinje fibers in culture (Fig. 4G). Consistent with a negative response to ET treatment (Fig. 4G), no hybridization signal of *Myf5* and *MRF4* was found in the myocardium (negative results are not shown). The data suggest that, although *myoD* is expressed at lower levels in a subpopulation of ET-induced and *bona fide* Purkinje fibers, expression of skeletal muscle-type myofibrillar proteins occurs in conduction cells without significant induction of other skeletal muscle transcription factors, such as Myf5 and MRF4.

DISCUSSION

Our previous studies of chicken embryonic hearts have identified the myocyte origin of Purkinje fibers (Gourdie *et al.*, 1995; Cheng *et al.*, 1999), and that the ET-signaling pathway induces the myocyte-to-conduction cell conversion (Gourdie *et al.*, 1998; Hyer *et al.*, 1999; Takebayashi *et al.*, 2000). However, little is known about transcriptional mechanisms governing the unique array of gene expression in conduction cells. This was addressed in this present study, focusing on the change in gene expression as cells convert from contractile to conducting types. We found that *bona fide* Purkinje fibers which down-regulate a cardiac muscle myofibrillar protein, cMyBP-C, up-regulate cardiac muscle-specific transcription factors, *GATA4* and *Nkx2.5*, at significantly higher levels compared to ordinary heart muscle cells. Similarly, ET-induced Purkinje fibers from embryonic cardiomyocytes in culture, dramatically down-regulate cMyBP-C expression, whereas expression of *Nkx2.5* and *GATA4* persist. In addition, *myoD*, a skeletal muscle transcription factor, was detected in ET-induced Purkinje cells, while *Myf5* and *MRF4* transcripts were undetectable in these cells. These results strongly suggest that the mechanism underlying down-regulation of cardiac muscle genes and up-regulation of skeletal muscle genes during conduction cell differentiation may be distinct from the transcriptional control seen in ordinary cardiac and skeletal muscle.

Most muscle proteins are transiently expressed in both skeletal and cardiac muscle during embryonic stages (reviewed in Lyons, 1994). An important exception is the expression of the MyBP-C gene family (Yasuda *et al.*, 1995): cardiac muscle cells express only the cardiac isoform (cMyBP-C), but not skeletal muscle isoforms, throughout development (Gautel *et al.*, 1998; Fougerousse *et al.*, 1998). Mutations in the cMyBP-C gene have been identified as the cause of chromosome 11-associated autosomal-dominant familial hypertrophic cardiomyopathy (Watkins *et al.*, 1995; Bonne *et al.*, 1995). We have previously shown that cMyBP-C, which is essential for normal heart muscle contractility, is down-regulated in Purkinje fibers (Gourdie *et al.*, 1998; Takebayashi-Suzuki *et al.*, 2000). The down-regulation of cMyBP-C is ET-dependent (Gourdie *et al.*, 1998) and is an early marker of Purkinje fiber differentiation from contractile myocytes (Takebayashi-Suzuki *et al.*, 2000). The data from the present study show that cMyBP-C down-regulation in differentiating Purkinje fibers occurs without significant transcriptional suppression of the cardiac muscle transcription factors, *Nkx2.5*, *GATA4*, or *Mef2C*. Rather, expression of *Nkx2.5* and *GATA4* appear to be up-regulated in Purkinje fibers.

Our studies have shown that expression of many Purkinje fiber marker genes as well as down-regulation of heart muscle-specific genes can be induced by ET both *in vitro* and *in vivo* (Gourdie *et al.*, 1998; Takebayashi-Suzuki *et al.*, 2000). In the present study, however, we found no significant change in expression of *Nkx2.5* and *GATA4* in ET-induced Purkinje fibers in culture, while both factors are up-regulated in *bona fide* Purkinje fibers. This is the first example of Purkinje fiber marker genes that do not respond to ET in culture, suggesting that an ET-independent pathway may play a role in regulation of a unique gene expression pattern in Purkinje fibers.

Our analysis of mRNAs does not rule out the possibility that posttranslational suppression of Nkx2.5 and GATA4 proteins may be associated with cMyBP-C down-regulation in conduction cells. However, higher levels of *Nkx2.5* and *GATA4* mRNAs in Purkinje fibers are consistent with the fact that Nkx2.5 and GATA4 cooperatively activate the

expression of ANF (Dorocher *et al.*, 1997; Lee *et al.*, 1998), which is highly expressed in both the atrium and the ventricular conduction system (Wharton *et al.*, 1988; Hansson and Forsgren, 1993). Our results are also consistent with the recent report (Schott *et al.*, 1998) that mutations in the human *NKX2.5* result in an inherited disease of an autosomal dominant atrial septal defect and atrioventricular conduction delays. The data, however, still do not explain the mechanism by which cMyBP-C is down-regulated in Purkinje fibers. Since the organization and sequence of the *cMyBP-C* gene, which comprises over 21 kilobase pairs and contains 35 exons, has been recently solved (Carrier *et al.*, 1997), detailed characterization of the gene, including cis-elements analysis, would be essential for obtaining the basis for further studies of the gene regulation work involved in conversion of myocytes to Purkinje fibers.

In contrast to uncertainty in *cis*-elements responsible for down-regulation of heart muscle genes in Purkinje fibers, a skeletal muscle-specific enhancer/promoter has been shown to direct specific gene expression in the peripheral conduction system. A 280-bp enhancer of the desmin gene (DES1) that contains several binding sites of myogenic transcription factors, including MEF2 and myoD, promotes high levels of expression in the skeletal muscle (Li and Paulin, 1991). The only exception is its activity in Purkinje fibers of the heart (Li *et al.*, 1993); mice transgenic for a reporter gene, *lacZ*, under the control of the DES1 promoter express β-gal in the peripheral conduction system throughout embryonic development and after birth. Furthermore, Mef2s, a cofactor of the myoD family, has been shown to be expressed in the heart (Edmondson *et al.*, 1994). Although these studies have suggested the potential role of the myoD family in Purkinje fibers, no member of the gene family has been identified in the heart. Because Purkinje fibers are an extremely minor population in the myocardium, detection of such factors perhaps needed a method that provides higher sensitivity than homologous cloning of a cDNA library from the total heart RNA. We solved this problem by enriching Purkinje fiber cells through experimental conversion of embryonic myocytes into conduction cells in culture with ET, as described above. Our immunohistochemistry and RT-PCR analyses have detected for the first time the myoD family members in ET-induced Purkinje fibers. The same antibodies and riboprobes also identified their expression in *bona fide* Purkinje fibers. However, since our data only show that epitopes and mRNAs encode a region identical to those of skeletal muscle *myoD* and *myogenin*, their definitive identity has to await complete sequencing of full-length mRNAs in the future.

The expression profile by itself does not determine the exact role of the myoD family members in inducing expression of skeletal muscle-type genes in ET-induced and *bona fide* Purkinje fibers. However, *myoD* has been ectopically expressed in the developing mouse heart (Miner *et al.*, 1992), giving rise to induction of skeletal muscle α-actin and two skeletal muscle-specific MyHCs, embryonic and perinatal, in late stage embryonic hearts. Interestingly, ectopic *myoD* fails to activate *Myf-5* or *MRF-4* (Miner *et al.*, 1992): the only myogenic counterpart induced in the transgenic mouse by *myoD* is *myogenin*. It has also been shown that the frog heart expresses low levels of *myoD*, but not myogenin and *MRF4*, although it is unclear which cell type expresses *myoD* (Jennigs, 1992). Coincident with the results, our present study shows that ET-induced Purkinje fibers express *myoD*, while *Myf5* or *MRF4* is undetectable. Thus, the transcriptional mechanisms of inducing skeletal muscle-type genes in conduction cells may be different from those functioning in skeletal muscle.

Since the transcriptional switch that is induced during Purkinje fiber differentiation from myocytes includes not only induction of skeletal muscle-type genes but also induction of neuronal genes and down-regulation of cardiac muscle genes, it is unlikely that a single "master" transcription factor controls all programs underlying the unique gene expression of Purkinje fibers. Consistent with this idea, our data demonstrate that, during and after conversion from heart muscle cells, Purkinje fibers express a unique myogenic transcription factor program which is distinct from ordinary cardiac or skeletal muscle. Our study would provide a basis to clarify the complex regulatory mechanisms involved in conduction cell development.

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